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ECOLOGY AND THERMAL INACTIVATION OF MICROBES
IN AND ON INTERPLANETARY SPACE VEHICLE
COMPONENTS

Thirty-sixth Quarterly Report of Progress

Order No. W-13411

January 1, 1974 - March 31, 1974

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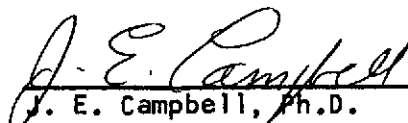
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Introduction

In the past several years, we have used the conventional pour plate method for assaying spores exposed to wet heat and dry heat at elevated temperatures. In this method an aliquot of a sample is diluted, plated, and incubated, and visible growing colonies are counted. The number of surviving organisms is calculated, and the results plotted on log paper: the number of viable organisms on the ordinate and the time of heat exposure on the abscissa. There are other methods for enumerating microorganisms but they are either time consuming or inadequate.

This Quarter we directed our efforts to exploring some of the uses of scanning electron microscopy in assessing changes that occur in spores exposed to wet and dry heat cycles. We feel this technique may be useful for directly enumerating microorganisms and also for determining viability by morphological characteristics. Scanning electron micrograph services have been provided under an agreement with Dr. P. S. Lin of the Radiobiology Division, Tufts University School of Medicine, Boston, Massachusetts. In this investigation, four Bacillus species and four nonspore-forming species of organisms were examined.

I. EXPERIMENTAL

A. Organisms

Four species of aerobic spore-forming organisms (Bacillus subtilis var. niger, Bacillus cereus, Bacillus megaterium, Bacillus

pumilus) and four nonspore-formers (Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, and Pseudomonas aeruginosa were selected.

1. Preparation of spores

The organisms were produced and harvested according to the method described (First Quarterly Report of Progress).

a. Dried spores (nonheat-treated)

From a spore stock suspension, approximately 1.0×10^9 spores/ml were air-dried through a 0.45 μ membrane millipore filter, and the pellet scraped with a sterile scalpel. This pellet was placed in a 5-ml screw-capped vial, and was stored in a 4 C refrigerator before shipment for scanning electron microscopy examination.

b. Dried spores (nonheat-treated and mechanically ground). The preparation of spores is similar to (a) except that after air-drying, the spores were ground by a mortar and pestle.

c. Dry heat

This experiment was carried out in the following manner: Approximately 1×10^9 spores/ml was dispensed into a stainless steel cup. The cups were arranged on circular shelves and placed in 206 mm x 300 mm tin cans. Each can contained four shelves of 30 cups each, for a total of 120 cups/can. The cans, lids, and contents

were dried in a vacuum oven for 90 min at 45 to 50 C (at 1.5-inch Hg pressure absolute). To increase the drying rate, the oven was purged with dry nitrogen every 10 min for the first 70 min, followed by five consecutive purges of nitrogen, with a vacuum cycle between each purge. After the drying process, the cans, lids, and contents were removed from the oven and cooled to about 30 C in the equilibration hood. In order for each can to reach the desired RH, different calculated amounts of water or P_2O_5 were placed in each can. The cans were sealed and removed from the equilibration hood. Heat treatments were applied at 113 C at various time intervals.

The can was opened with an automatic can opener, and the cup, containing the spores, was scraped and dispensed in 5-ml screw-cap vials containing a 2% glutaraldehyde fixative.

d. Wet heat

Bacillus subtilis var. niger spores in phosphate buffered water were dispensed in 3-ml amounts into each of 16 x 125 mm screw-cap tubes to give about 1×10^{10} spores/ml. These tubes were immersed in a silicone oil bath and heated at 90 C for various time intervals. The tubes were then removed and cooled for 15 min at 4 C. Each sample was centrifuged, the decantate was poured, and 2% glutaraldehyde fixative

was thoroughly mixed with the pellet, and poured in a 5-ml screw -cap vial.

2. Preparation of bacterial cells

Actively-growing cultures of all organisms were grown in flasks of trypticase soy broth. The medium was continually agitated with a magnetic stirring bar and incubated for 18 to 24 hr at 35 C. After incubation, the cells were washed twice with double distilled water and centrifuged, and the supernatant discarded. The remaining pellet was resuspended and mixed thoroughly in a 2% glutaraldehyde fixative, and placed in 5-ml screw-cap vial.

II. RESULTS

Scanning electron micrographs of the spores and bacterial cells are shown in Figures 1 to 30. The groups of species and the type of treatment are given below:

A. Bacillus subtilis var. niger spores

Figures 1 and 2 show air-dried spores. The surface structures showed angulation at both ends. In some cases, spores were longer than others. Similar surface features are evident on dry-heat-treated spores heated at 113 C over P_2O_5 (Figures 3 and 4), and spores dry-heated at 113 C for 60 min under headspace moisture of $0.15 \mu\text{g H}_2\text{O/ml}$ (Figures 5 and 6). However, spores heated for 30 min in phosphate buffer showed general smoothness and loss of angulation on both ends (Figs. 7 and 8). Swelling of spores is also evident (Fig. 8). Figures 9 and 10 show spores heated at 90 C in

phosphate buffered water for 240 min. Figures 7-10 suggest that spores absorb water. The increase of water uptake caused an increase in osmotic pressure, resulting in the weakening of the general spore structures, and eventual bursting of cells.

Similar features were observed on spores ground by a mortar and pestle (Figures 11 and 12) as compared to dried spores (unheated) and dry-heat treated spores.

Dried spores (unheated) of Bacillus pumilus (Figures 13 and 14), Bacillus cereus (Figures 15 and 16), and Bacillus megaterium (Figures 17 and 18) showed dissimilar surface features. For example, Bacillus pumilus spores appeared to be more slender, whereas Bacillus megaterium spores were smoother and wider. Of the four Bacillus species examined, only Bacillus subtilis var. niger spores (Figures 1-18) showed angulation on both ends. Figures 19 and 20 show Bacillus subtilis var. niger in vegetative form. The characteristic rodshape appearance found in the genus, Bacillus, is evident. The difference in cell length probably is attributed to their growth phase. The same vegetative cells autoclaved for 15 min at 121 C showed varying shapes and masses of cell debris.

Grape-like features that are characteristic of the genus, Staphylococcus, are shown in Figures 23-26. Rod-shape surface features of Escherichia coli and Pseudomonas aeruginosa are shown in Figures 27-30.

In general, the initial study on scanning electron microscopy looked promising because it provided some concise description

of the surface morphology of viable and nonviable organisms.

We feel that this method would be very useful in collaborating results assayed by the conventional methods.

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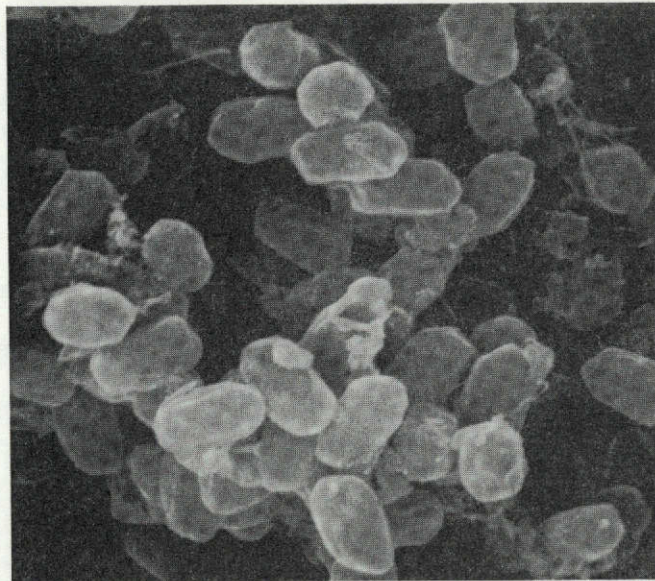


Figure 1. Bacillus subtilis var. niger spores
(10,000 X magnification)

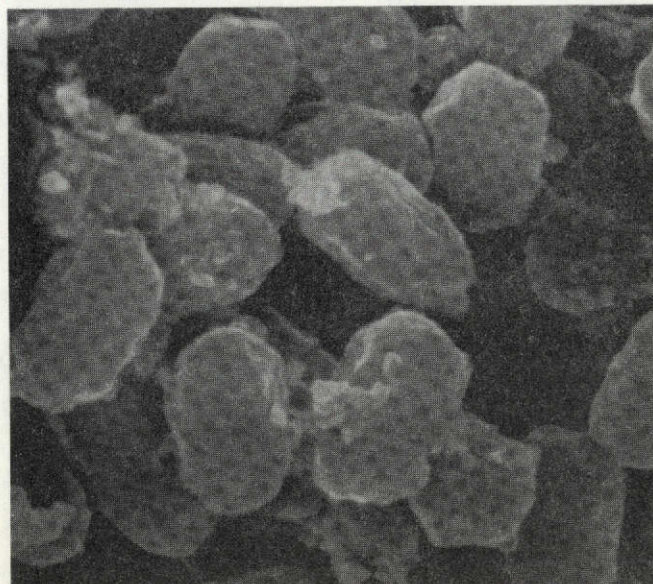


Figure 1. Bacillus subtilis var. niger spores
(20,000 X magnification)

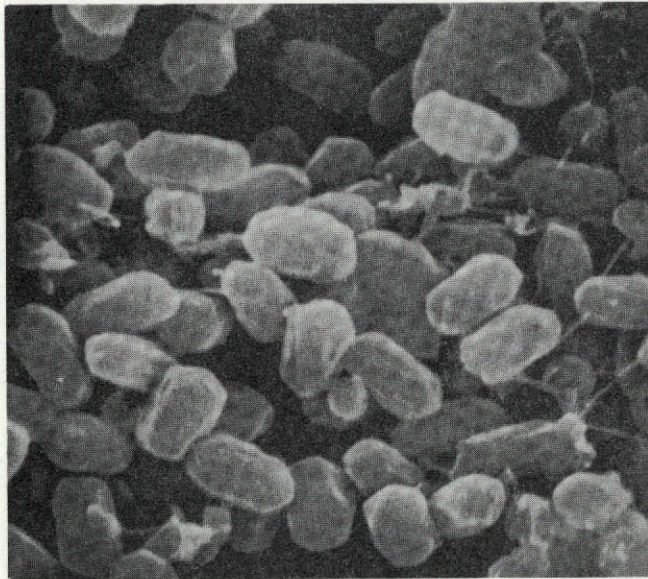


Figure 3. Bacillus subtilis var. niger spores heated over P_{205} at 113 C for 90 minutes (10,000 X magnification)

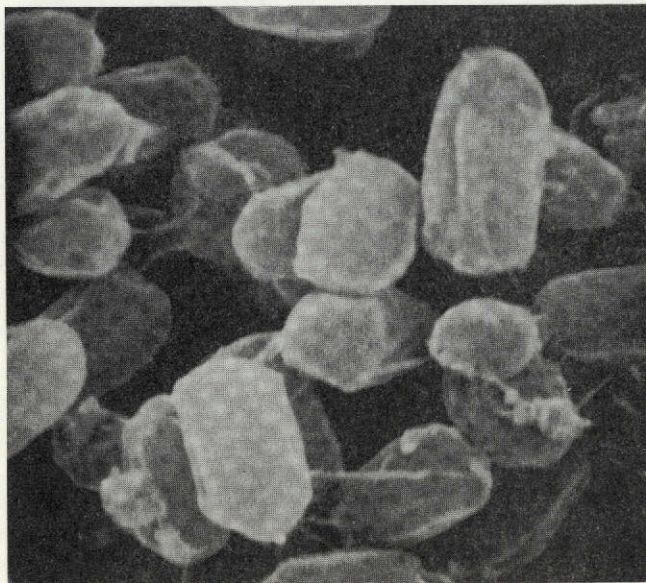


Figure 4. Bacillus subtilis var. niger spores heated over P_{205} at 113 C for 90 minutes (20,000 X magnification)

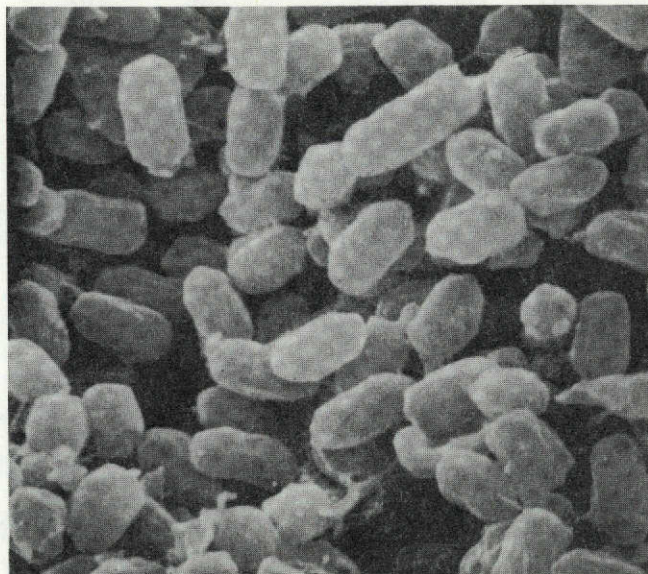


Figure 5. *Bacillus subtilis* var. *niger* spores heated for 60 minutes at 113 C under headspace moisture of 0.15 μ g H_2O/ml (10,000 X magnification)

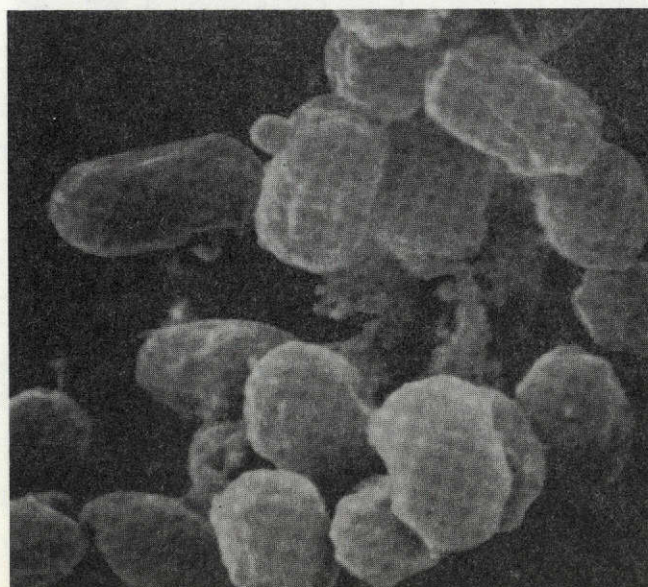


Figure 6. *Bacillus subtilis* var. *niger* spores heated for 60 minutes at 113 C under headspace moisture of 0.15 μ g H_2O/ml (20,000 X magnification)

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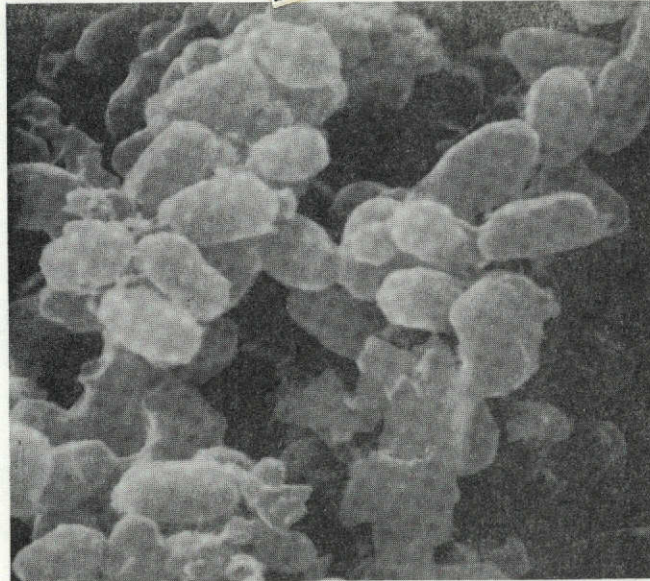


Figure 7. *Bacillus subtilis* var. *niger* spores heated in buffered water for 30 minutes at 90 C (10,000 X magnification)

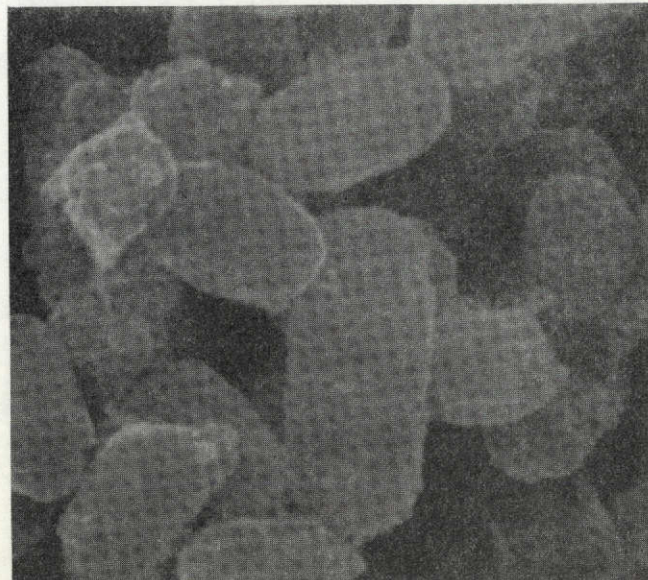


Figure 8. *Bacillus subtilis* var. *niger* spores heated in buffered water for 30 minutes at 90 C (20,000 X magnification)

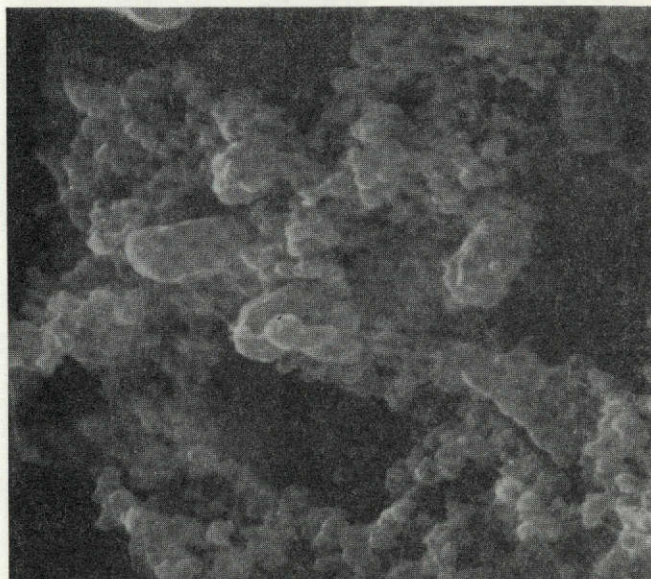


Figure 9. Bacillus subtilis var. niger spores heated in buffered water for 240 minutes at 90 C (10,000 X magnification)

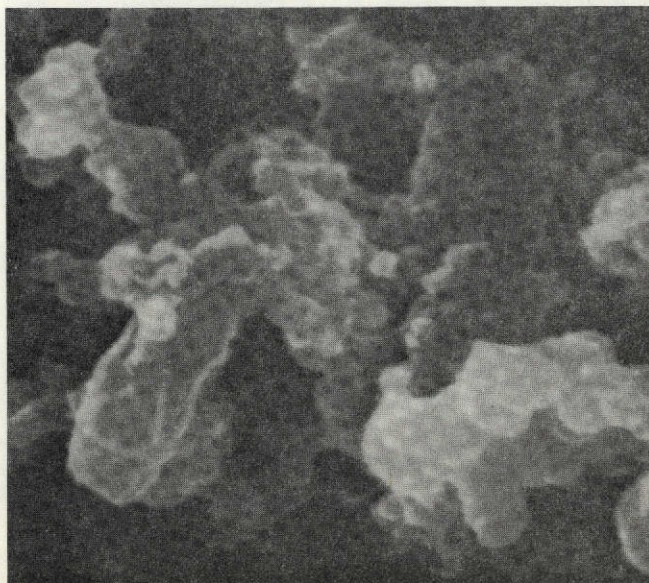


Figure 10. Bacillus subtilis var. niger spores heated in buffered water for 240 minutes at 90 C (20,000 X magnification)

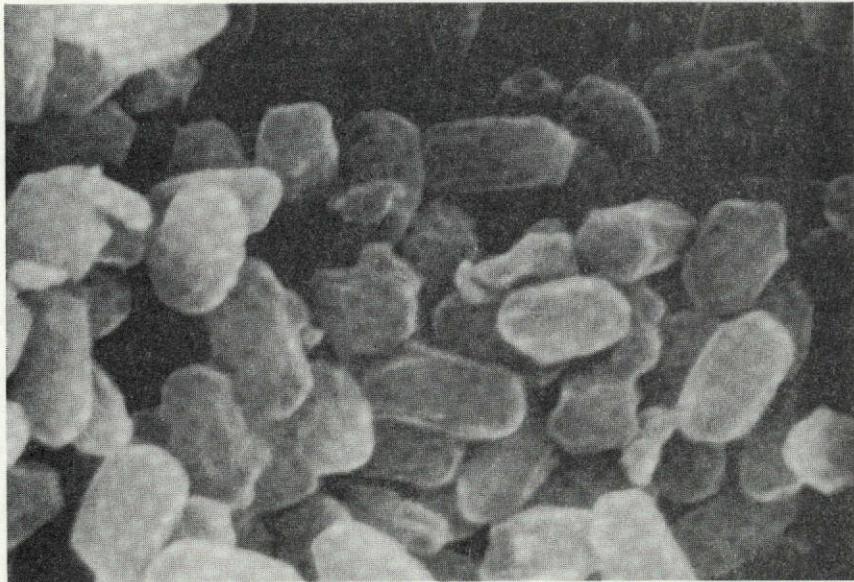


Figure 11. Bacillus subtilis var. niger spores -
dry and mechanically ground (10,000 X magnification)

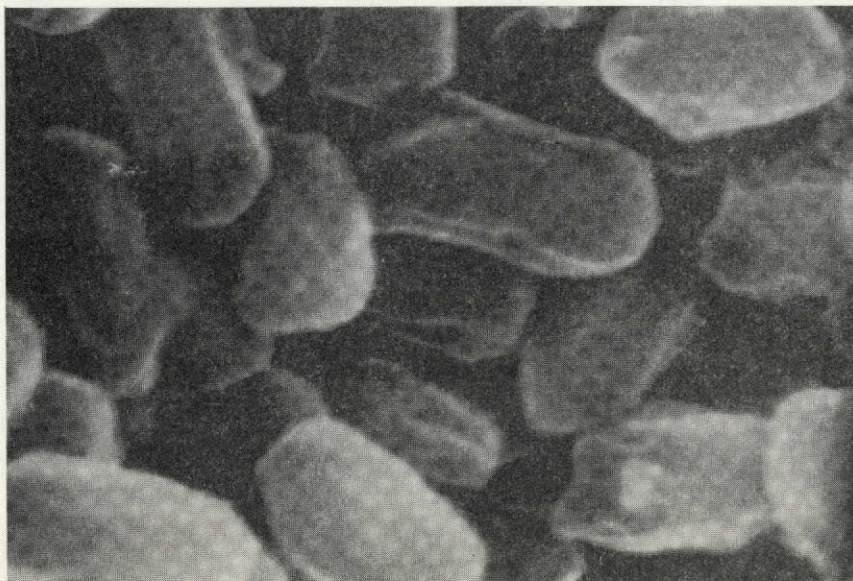


Figure 12. Bacillus subtilis var. niger spores -
dry and mechanically ground (20,000 X magnification)

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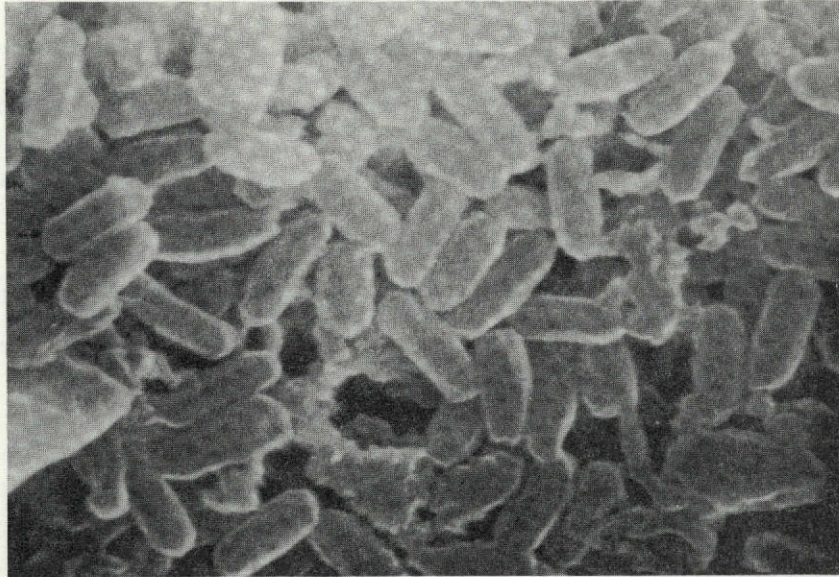


Figure 13. *Bacillus pumilus* dry spores
(10,000 X magnification)

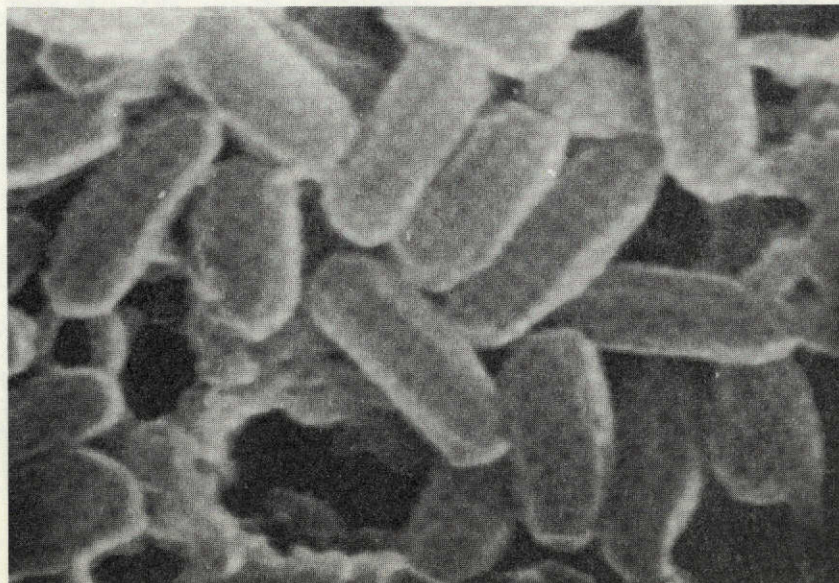


Figure 14. *Bacillus pumilus* dry spores
(20,000 X magnification)

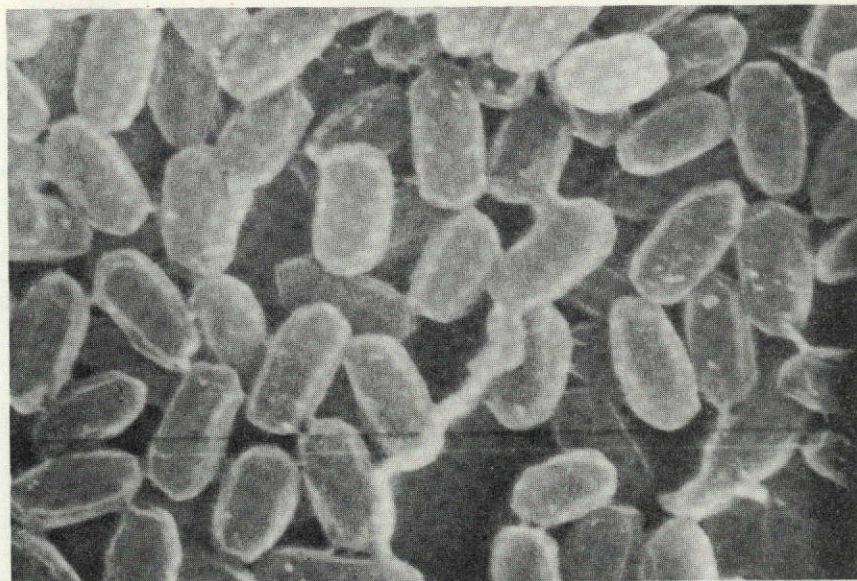


Figure 15. Bacillus cereus dry spores
(10,000 X magnification)

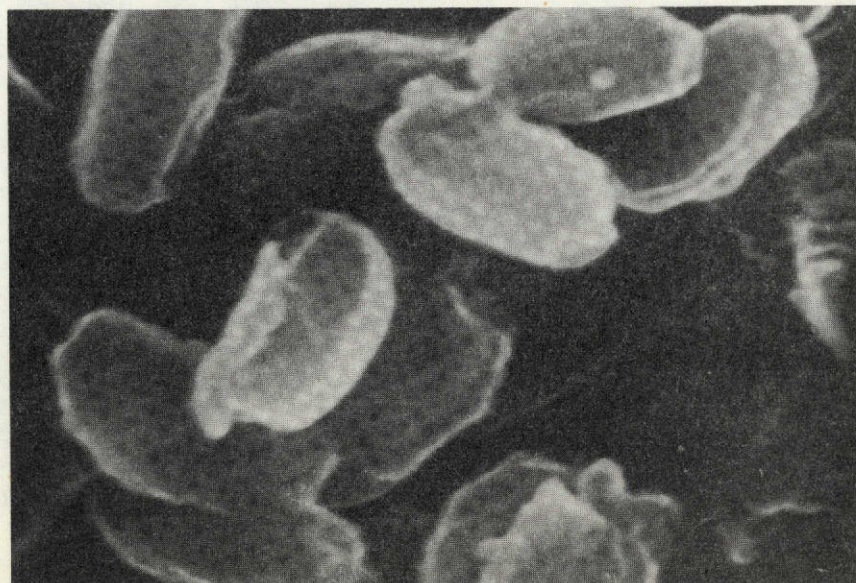


Figure 16. Bacillus cereus dry spores
(20,000 X magnification)

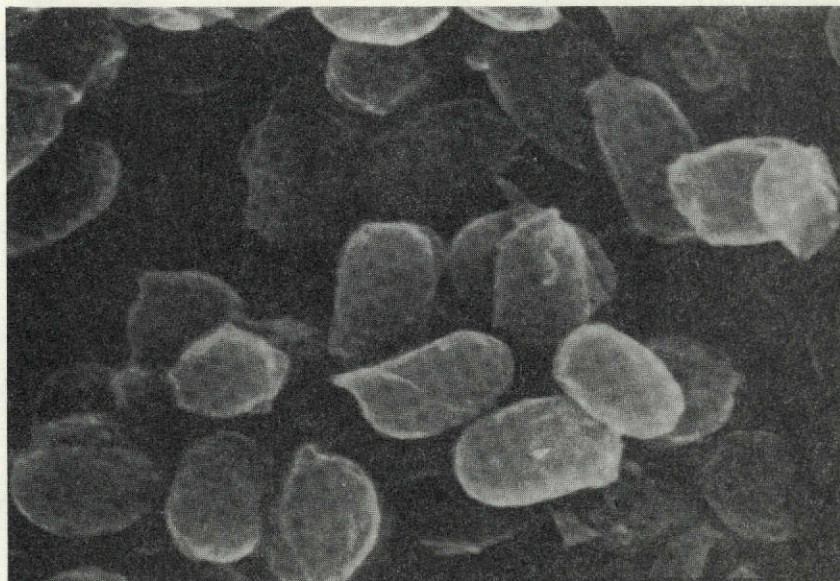


Figure 17. Bacillus megaterium dry spores
(10,000 X magnification)

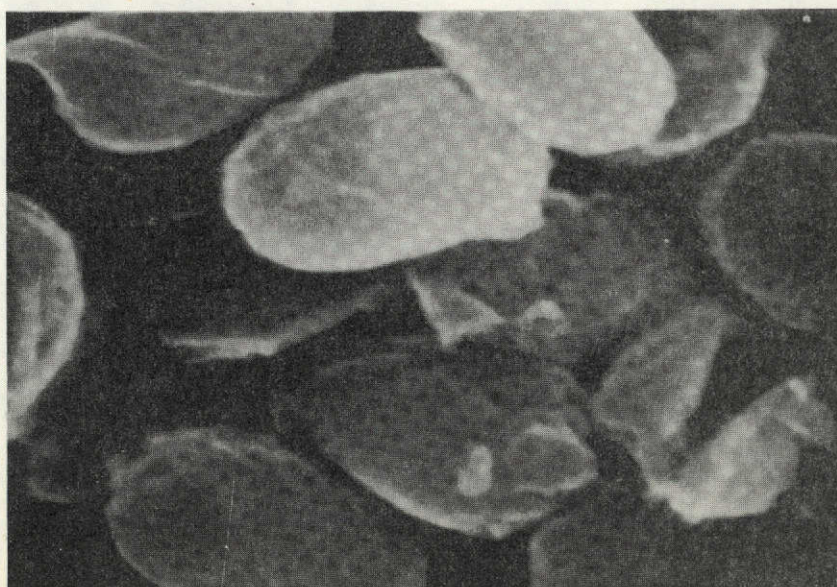


Figure 18. Bacillus megaterium dry spores
(20,000 X magnification)

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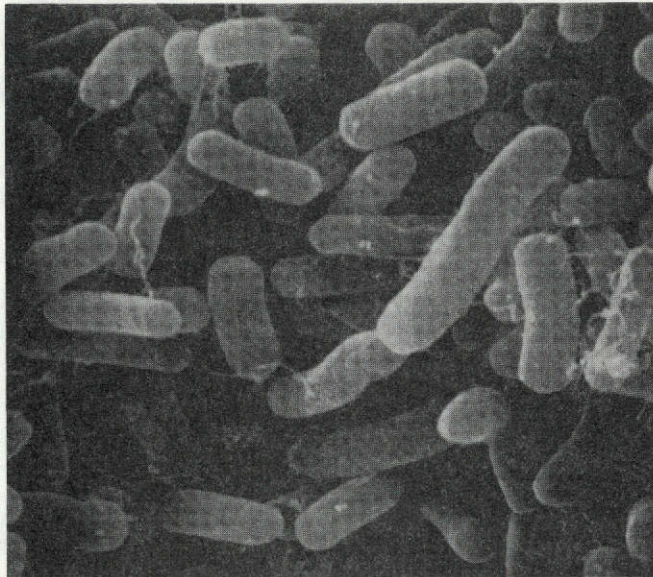


Figure 19. Bacillus subtilis var. niger
vegetative form (10,000 X magnification)

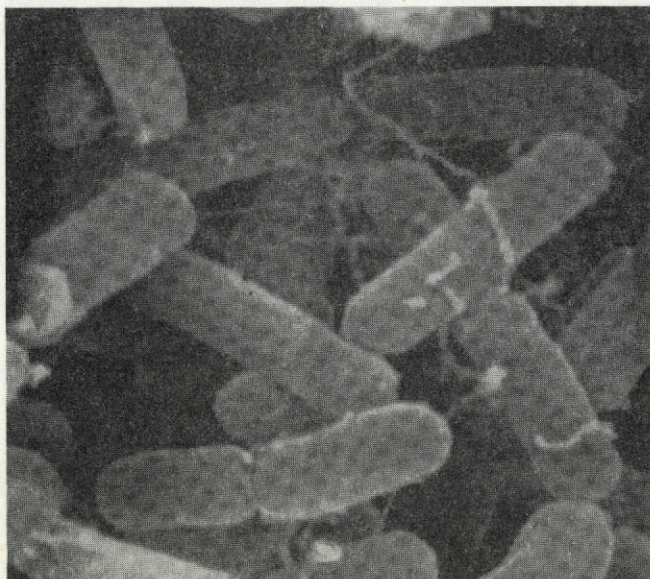


Figure 20. Bacillus subtilis var. niger
vegetative form (20,000 X magnification)

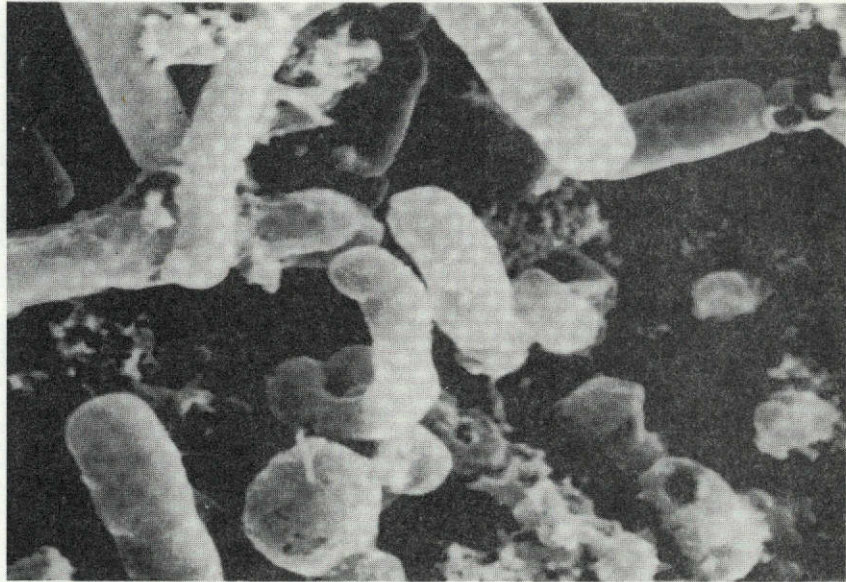


Figure 21. Bacillus subtilis var. niger - vegetative cells autoclaved at 121 C for 15 minutes (10,000 X magnification)



Figure 22. Bacillus subtilis var. niger - vegetative cells autoclaved at 121 C for 15 minutes (20,000 X magnification)

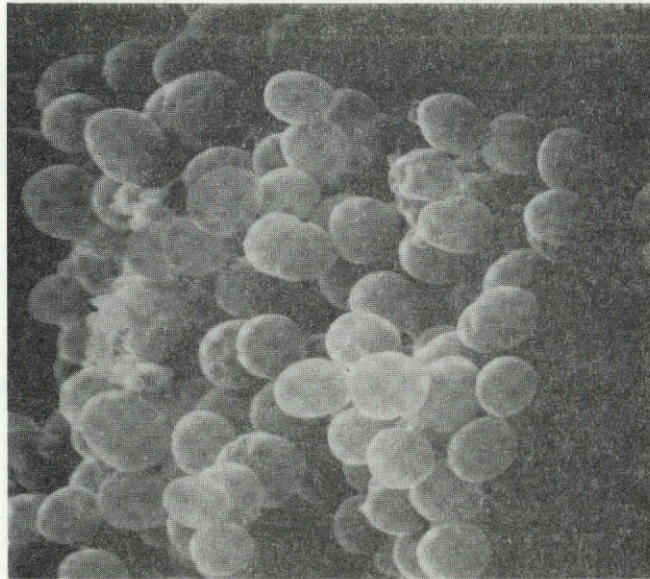


Figure 23. Staphylococcus aureus (10,000 X magnification)

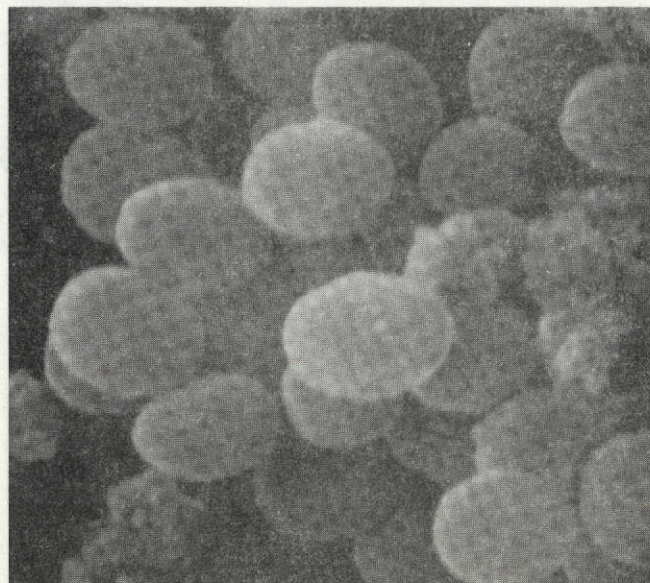


Figure 24. Staphylococcus aureus (20,000 X magnification)

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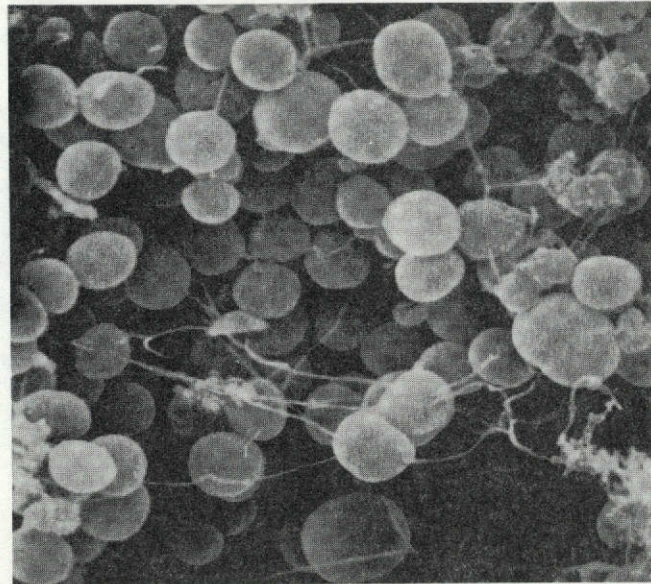


Figure 25. Staphylococcus epidermidis (10,000 X magnification)

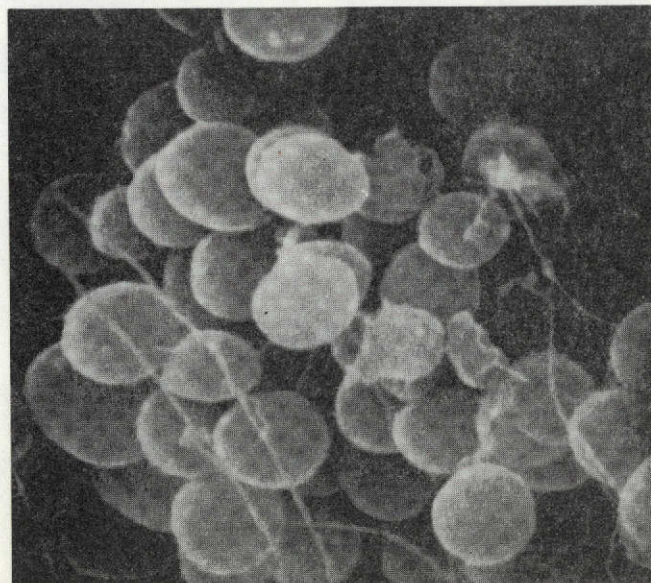


Figure 26. Staphylococcus epidermidis (20,000 X magnification)

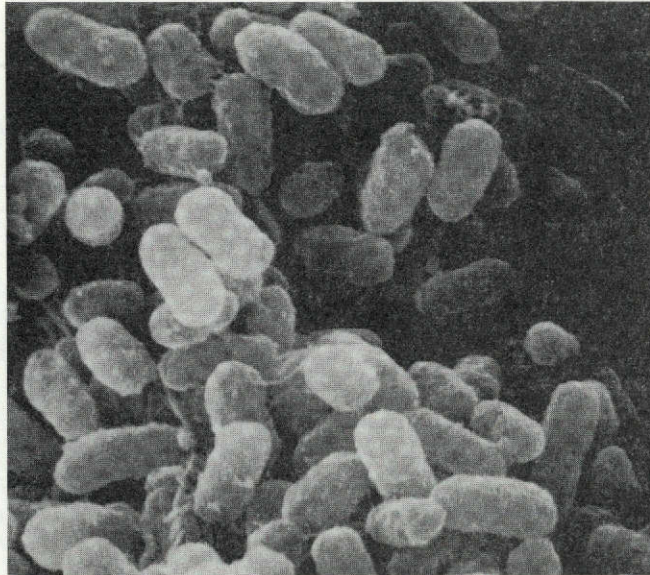


Figure 27. Escherichia coli (10,000 X magnification)

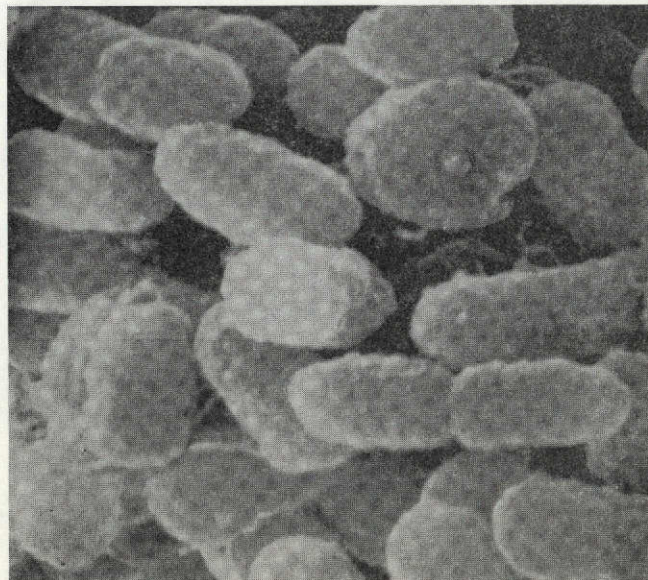


Figure 28. Escherichia coli (20,000 X magnification)

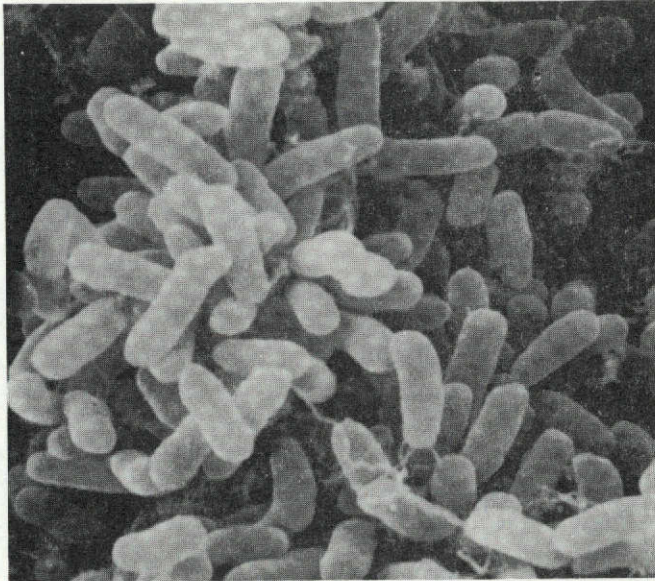


Figure 29. Pseudomonas aeruginosa (10,000 X magnification)

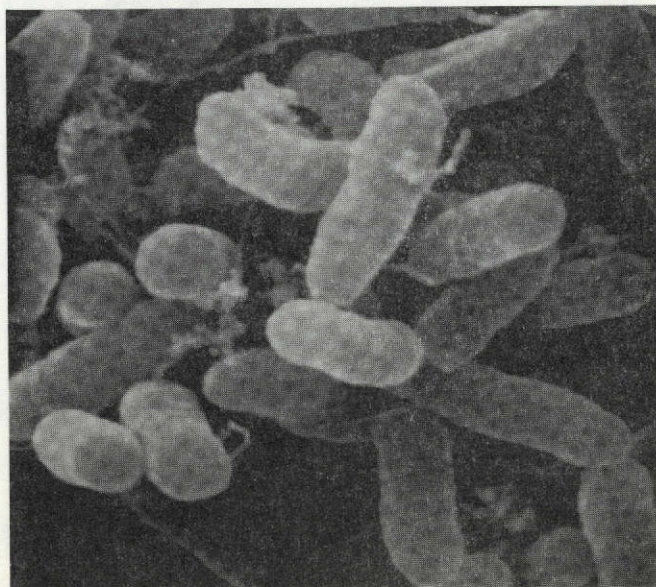


Figure 30. Pseudomonas aeruginosa (20,000 X magnification)